SPONTANEOUS MUTATIONS OF BACTERIA

M. DELBRÜCK
Vanderbilt University, Nashville, Tennessee

The study of bacterial mutability is our only avenue of approach to problems of heredity in bacteria. This approach may be followed along two lines. Along one line one focuses his attention on the phenotypic differences between two strains which differ by one mutational step, in the endeavor to trace the reaction chains from the phenotype back to the gene. This is the analogue to physiological genetics and is exemplified, for instance, by E. H. Anderson's (1) comparative study of the physiology of virus-resistant bacterial mutants.

Along the other line one focuses his attention not on the mutant and its phenotype but on the mutational step, specifically on the rate at which it occurs under some standard conditions. At first sight such a study seems rather aimless because the use of determining this or that mutation rate is not apparent. Studies of this kind can, however, become of interest in two ways. It may be found that the mutation rate can be controlled by cultural conditions. Such a finding might give us a lead as to the nature of the mutational process. Investigations of this sort have not yet been reported in the literature but very likely could be undertaken with great profit. Another use of the study of mutation rates lies in the possibility of considering the mutational pattern of a strain of bacteria and of its mutants. Attempts along these lines have been reported by Luria (2), by E. H. Anderson (1), and by Demerec and Fano (3). It is quite evident from these papers that the same mutational step can be identified in organisms which differ by one or more mutational steps of a different kind. This use of mutation rates, and of mutational patterns, may prove to be an invaluable substitute for Mendel's experiments for purposes of factor analysis in bacteria.

The main problems which face the experimenter along this line are:

1. The estimation of the number of mutants in a given culture.
2. The evaluation of the mutation rate.
3. The differentiation between mutations which may lead to phenotypically similar forms, and conversely the identification of the same mutation if it occurs repeatedly.

Before discussing these topics, I would like to make a few general remarks about methods of detecting and culturing mutants of bacteria. Some of these remarks are also pertinent to the study of mutations of viruses.

1. POPULATION DYNAMICS AND MUTATION PATTERNS

We will assume that we start from a stock strain with fairly constant properties. Actually, a variety of mutations will occur during the subculture of such

a strain. The strain will, therefore, not be pure in the genetic sense, but will represent a population which is in approximate equilibrium with its mutants. The equilibrium will be determined by three factors, namely, the forward mutation rates, a_1, a_2, etc., which lead away from the normal type, the reverse mutation rates, b_1, b_2, etc., and the selection rates, s_1, s_2, etc.[1]

We will measure mutation rates in units of mutations per bacterium and per time unit, and selection rates by the difference between the growth rate constants[2], k_N, of the normal type, and k_M, of the mutant type,

$$s = k_N - k_M$$

The populational equilibrium between the normal type and its mutants will depend on the cultural conditions. If the cultural conditions are altered the equilibrium will in general shift, and the shift may or may not be reversible when the strain is returned to the standard conditions.

Certain conditions must be fulfilled by the mutation and selection rates for the equilibrium to be reasonably stable. For instance, if a certain forward mutation rate, a_i, is high, it must be balanced either by a high selection pressure against this mutant, or by a high reverse mutation rate.

If the mutant is balanced by adverse selection then the equilibrium proportion of mutants is given by[2]

$$M/(M + N) = a/s$$

and this fraction must be small compared to unity. If a sub-culture is started from a single normal bacterium, equilibrium will be approached with a rate equal to $s - a$. Since the mutation rate must be small compared to the selection rate, the approach to equilibrium is essentially determined by the selection rate.

If the mutant is balanced by reverse mutation then the equilibrium proportion of mutants is given by

$$M/N = a/b$$

Since this ratio must again be small, the reverse rate must be large compared to the forward rate. The rate of approach to equilibrium in this case is determined by the sum of the forward and reverse mutation rates, $a + b$, that is, essentially by b.

If the forward mutation rate is very small it need not be balanced by selection pressure against it or by reverse mutations because the mutant type will in general be eliminated on each sub-culture when small samples are used for the sub-culture.

It may sound strange that the conditions of equilibrium should differ for small and high mutation rates. Actually, of course, there is no difference in the

[1] Strictly speaking, we should also include among the factors which determine the population equilibrium all the rates of mutation of the mutants. However, most of these second-step mutations will cause only small shifts of the equilibrium. If a particular step should be fast the two steps can be lumped together as one mutation.

[2] See Appendix I at the end of this paper for definition of growth-rate constants and for mathematical derivations.

equations which determine the average values of mutants in a very large number of cultures in these two cases. However, for small mutation rates the fluctuations in these numbers become the dominant feature. Suppose we are dealing with a mutation rate of 10^{-8}. If we were dealing with millions of similar cultures, or if we would sub-culture one culture many million times we would be likely at one time or another to transfer a great many mutants, and from then on the culture would contain a high proportion of mutants. In actual experiments, however, we are not likely to encounter these rare events which are included in the ideal average.

When studying the mutational pattern of a stock strain with reasonably stable properties we may therefore expect to find, on the one hand, mutants which occur with high frequency but grow more slowly than the normal type or revert quickly, and, on the other hand, mutants which occur with low frequency and which may or may not be at a selective disadvaneage due to slow growth or reverse mutations. These expectations are borne out by the general experience that stable and vigorous mutants of stock strains always occur with very low frequency.

The same is not true of the mutational pattern of the mutants themselves. The only prediction that can be made regarding the patterns of the mutants is that mutants which occur frequently and are not at a selective disadvantage must have a high reverse rate. Such mutants are, of course, of great importance for the study of the mutational pattern of the normal type, but it stands to reason that their study presents special difficulties.

2. THE ESTIMATION OF THE NUMBER OF MUTANTS

We may distinguish between three methods of detecting mutants in cultures of bacteria.

The first method consists in plating out a sample of the normal culture so as to obtain isolated colonies. The mutants may then be picked out either simply by inspection of the colonies or by special tests of a large number of colonies selected at random. The first alternative is used for picking mutants which affect the morphology or pigmentation of colonies. Using the technique of Spiegelman, Lindegren and Hedgecock (4), it may also be found possible to pick by this method mutants which affect the fermentative properties of the strain. The second alternative has been applied by Roepke, Libby and Small (5) to obtain growthfactor-deficient mutants.

The second method is to subject the normal culture to a destructive treatment, such as irradiation with ultra-violet light (Maisel-Witkin unpublished) or exposure to penicillin (Demerec unpublished). If the normal population contains mutants with somewhat heightened resistance to these destructive agents, then these resistant mutants will be present in higher proportion in the fraction of bacteria which survives the treatment. In the ideal case the surviving fraction of bacteria will consist entirely of mutants with increased resistance. The method

amounts, in such a case, to a direct isolation of the mutants, but of course not all mutants with increased resistance present in the original culture are isolated by this method, because most of them will be killed by the treatment. Consequently, it is quite difficult to determine the fraction of this mutant type in the normal culture.

The third method is to use a treatment which will eliminate all normal bacteria while leaving all individuals of certain mutant types unaffected. The only treatment known that will give such a clear-cut segregation of certain mutant types is exposure to an excess of virus particles which are active on the normal strain of bacteria. Luria has shown that a similar principle can be used to isolate mutants of *viruses*, by plating a large number of virus particles with a strain of bacteria resistant to the normal type of virus of the stock used. Such stocks may contain mutant viruses which will attack and multiply at the expense of the bacterial strain.

Mutants must fulfill certain conditions to permit their maintenance and study. In general, reverse mutation rates must not be so high that the normal populational equilibrium is reached in a single sub-culture. In growth factor mutants the conditions are more stringent because even a very low reverse mutation rate will invalidate the test of mutation.

In virus-resistant mutants the conditions for maintenance are less stringent. Here the reverse mutants are automatically eliminated if plating is done in the presence of excess virus. If the reverse rate is rather high the mutant may become extinct in colonies starting from a single resistant bacterium. The chance for this to occur is $b/(1 - b)$, where b is the reverse rate per generation. The proof for this statement is given in Appendix II.

3. MUTATION RATES

In the past there has been much confusion about the concept of mutation rate. Many authors have simply divided the number of mutant bacteria found in a culture into the total number of bacteria and have called this fraction the mutation rate. It is obvious that the fraction of mutants is a poor measure of the mutation rate, since the number of mutants depends on two factors, namely, the number of mutations occurring in the culture and when they occurred. A mutation which appeared several generations back will be represented, at the time of the counting, not by one mutant, but by a sizeable clone of mutants.

First of all, we must define a mutation rate in such a fashion that it becomes a characteristic of each bacterium and expresses a property of this bacterium which can be measured (6). We define the mutation rate, therefore, as the probability of a bacterium to mutate during a given time unit under some specified physiological conditions. The time unit, of course, must not be greater than the lifetime of the bacterium. This conception of a mutation rate is quite analogous to the concept of probability of decay of a radio-active atom during a time unit.

In a growing culture of bacteria, mutations will occur with a frequency per time unit which increases in proportion with the size of the population. At any arbitrary moment, therefore, various-sized clones of mutants will be present in the population. Compare, for instance, clones of size 64 with those of size 128. Clones of these sizes originated in mutations which occurred respectively 6 or 7 generations back. Six generations back twice as many normal bacteria were present as seven generations ago. Clones of size 64 should therefore be twice as frequent as those of size 128. On the other hand, the average number of *mutants* belonging to clones of size 64 should just equal that belonging to clones of size 128. By logical extension one finds that the average number of mutants in a culture, say, 30 generations old, should fall into 30 groups of equal size, each group containing mutants of the same clone size. This statement, while correct, is not applicable to any real experimental situation, because the large clones, which contribute a large portion to the average, are so rare that they will not likely be found in a limited number of trials. The likely average number of mutants in a culture must therefore be calculated with omission of the contributions from the large but rare clones.

The likely average fraction of mutants thus turns out to be smaller than the ideal average fraction. The ideal average fraction would be equal to the rate multiplied by the time since the start of the culture. The likely average, on the other hand, is obtained by measuring the time, not from the start of the culture but from the time when the culture had reached a size at which a mutation becomes likely. Thus, for a mutation rate of, say, 10^{-6} per generation, we should count the time from the moment that the culture has reached a population size of about 10^6. If our observation is made when the population has reached a total number of 10^9, the time to be used would be 10, instead of 30, the number of generations since the start of the culture. Our correction in this case amounts to a factor of three. If the mutant grows more slowly than the normal type we must use a further correction to take into account that the mutants have not multiplied at the same rate as the normals.

The determination of the mutation rate from the number of mutants present at any given time suffers from another complication, namely, the very large fluctuations of the number of mutant bacteria in a series of similar cultures. It is easy to see why the fluctuations must be large. As we have seen above, the mutant bacteria, on the average, stem in equal numbers from all preceding generations. If the mutation rate is small, mutations will occur with any reasonable probability only during the last few generations, say the last ten generations as in the example above. Now the quantity which is subject to normal fluctuations is not the number of mutants but the number of mutations, in each generation. In the example cited one-tenth of the mutants will, on the average, be due to one mutation which occurred ten generations back, another tenth will be due to two mutations which occurred nine generations back, etc. It is clear that the one and two, etc., mutations are subject to large fluctuations. The net fluctuation in the final

number of mutants is therefore very appreciable, even if the number of mutants is quite large.

To make matters worse, the customary recourse to using large numbers of similar cultures for determining the average number of mutants is here of little use, because the more cultures we use the more likely we are to have a mutation occurring at a very early stage in the development of one of the cultures. Such an event is like hitting the jackpot; it will give us an erratic number of mutants and a most undesirable increase in the fluctuations.

To sum up this matter, we may say that the determination of the mutation rate from the number of mutants, even if we can measure the number accurately, is necessarily a statistically inefficient procedure.

4. REVERSE MUTATIONS

I should like to add a few remarks about the problem of studying reverse mutations. As has been pointed out before, reverse mutations, even if they occur frequently, do not interfere with the detection of forward mutations in mutations which affect the reaction of bacteria to viruses. While this is an advantage to the study of the forward rate it is a decided disadvantage in determining the reverse rate. At present we do not know of any generally applicable method for the determination of mutation rates from virus resistance to virus sensitivity.

Just the opposite of what has just been said is true of mutations which involve the acquisition of growth-factor requirements. These can be discovered only if the reverse rate is exceedingly small. However, if the reverse rate is small enough to permit the cultivation of the forward mutant then the reverse rate, even though it be very small, can be measured with reasonable accuracy. This is quite apparent from the work of Roepke, Libby and Small (5). Their method of detection of reverse mutants is to culture the factor requiring mutant in a basal medium to which a sub-optimal amount of the growth factor has been added. If reverse mutation occurs it shows by delayed full growth in this medium.

Now the mutants selected by any of the methods mentioned may differ from the normal in more characters than the one used to select it. Specifically, E. H. Anderson (1) has shown that many of the virus-resistant mutants are also mutants deficient in growth factors. In these special cases, therefore, the two methods might advantageously be combined; that is, one may use the virus resistance for the isolation of the forward mutants and the growth factor requirements for the detection of reverse mutation.

5. IDENTIFICATION AND DIFFERENTIATION OF MUTANTS

The chief interest of a study of a hypothetical pair of forward and reverse mutations would lie in ascertaining whether or not true reverse mutations do occur. It is difficult to decide whether the revert strain is identical with the original one. Theoretically, this implies the comparison of the original with the presumed revert strain in all measurable characteristics. In practice one has to

restrict himself to a selection of some fairly comprehensive tests. Tests with viruses are among the easiest.

The following example may serve as an illustration of the method. Two mutant strains of a bacterial strain (*E. coli* "B") had been isolated, one by using virus T1 as selecting agent, the other by using T5. These strains may be referred to as B/1 and B/5, respectively. Each of these strains was then plated with seven different viruses, T1 to T7, and with each virus they were tested in two ways, once by plating a large number of the bacteria (about 10^8) with an excess of virus particles, and once by plating with about 100 virus particles. The results are given in Tables I and II.

TABLE I

PLAQUES OBTAINED WHEN PLATING LOW TITER STOCKS OF VIRUSES ON B/1 AND B/5

	T1	T2	T3	T4	T5	T6	T7
B/1	0	183	118	146	0	47	59
B/5	0	160	106	172	0	44	64

Table I gives a comparison of the plaque counts obtained with these two bacterial strains for the seven viruses. It will be seen that both strains are resistant to both T1 and T5, and that they give equal plaque counts with all the other viruses. The two strains, therefore, agree in their resistance pattern.

At this point, however, we must inquire somewhat more closely into the nature of the mutations which make bacterial strains resistant to one or another group of viruses. It used to be believed that these mutations fall into two classes: On the one hand, there were thought to be mutations which completely alter the sensitivity pattern of the bacterial strain, and which in general also alter the morphology or other characteristics of the colony of the strain. These are the well-known "dissociative" changes from smooth to rough, etc. On the other hand, there were, it was believed, highly specific changes towards resistance to only one, or to a group of closely related viruses. These conceptions are due chiefly to Burnet (7), and the idea of classifying viruses by cross-resistance tests goes back to an important paper by Bail (8).

However, recent work has indicated that these conceptions are not tenable (9). There is no clear-cut distinction between mutations with generalized effects and those which cause nothing but resistance to a group of similar viruses. Specifically, it has been found that the classification of viruses by cross-resistance tests does not at all lead to a satisfactory grouping. Such classifications bring together viruses which are quite unrelated on other criteria, and they separate viruses which, on all other criteria, are most intimately related. The most important among these other criteria are the morphology as revealed by the electron microscope and cross-inactivation tests with specific antisera.

On second thought, we should not be surprised by these findings. Once it is granted that the mutations in question are not induced by the virus but are spontaneous, it seems understandable that one mutation may affect the sensitivity of the bacterium to quite unrelated viruses. At present, we do not know what makes a bacterium sensitive or resistant to a virus. We can imagine that certain substances must be elaborated whose presence permits the virus to grow in the bacterial cell. It stands to reason that a variety of genetic changes could interfere with the network of synthetic processes in such a fashion that the bacterium becomes unsuitable for the growth of the virus. Furthermore, resistance to two unrelated viruses in this picture means that there is a tie-up between the synthetic chains which lead from a gene to the substances required by these two viruses, respectively. The viruses themselves need have no similarity with each other. These ideas have been explained more fully in a recent paper by E. H. Anderson (1).

While we thus lose the cross-resistance test for the classification of the *viruses*, we can still retain the virus-sensitivity tests for the classification of *bacteria*, as in Table I. Tests of this kind have been used for the classification of naturally occurring strains of many species of bacteria. On the whole, the "typing" of an unknown strain by its virus-sensitivity pattern seems to be about as satisfactory a method as any other, and it seems to agree with serological typing.

Here, however, we are more interested in the possibility of classifying, not arbitrarily selected wild strains, but the family of mutants of one strain. With how much assurance can we here recognize identical mutants by their sensitivity pattern? An important piece of evidence on this point comes from the work of Demerec and Fano (3). These authors find that the same sensitivity pattern may be reached either in two mutations or in one mutation. Is the end product here genetically alike in the two cases, or only phenotypically? If the first, we would have to assume the existence of coupled mutations; if the latter, we might hope to identify phenotype differences by further tests, either with other viruses, or by physiological studies. Until such studies have been carried much further we can not form an opinion on the merits of the sensitivity tests for purposes of classification.

TABLE II

RESISTANT COLONIES OBTAINED WHEN PLATING HIGH TITER STOCKS OF VIRUSES ON B/1 AND B/5

	T1	T2	T3	T4	T5	T6	T7
B/1	Inf	4	233	275	Inf	38	518
B/5	Inf	14	200	250	Inf	20	600

Table II gives the number of resistant colonies obtained when plating these two strains with any of the seven viruses. Both B/1 and B/5 give complete growth when plated with high titers of T1 or T5, since they are resistant to these viruses. With the other viruses they give a fairly characteristic number of mutants, and it will be seen that the number of mutants obtained from B/1 is in all cases quite similar to the corresponding number obtained from B/5. What we are here comparing is really a portion of the mutational pattern of these two strains, and the parallelism might be considered strong evidence for the similarity, if not identity, of the strains.

Again, however, the results obtained by Demerec and Fano (3) should make us cautious before accepting this similarity of the mutational pattern as evidence of genetic similarity. In very extensive tests these authors could show that the mutational pattern is remarkably similar in bacterial strains which differ by one or two mutational steps. Similar results were found by Luria (2) and by E. H. Anderson (unpublished). Since, therefore, the available evidence shows that mutational patterns may be similar in strains with known differences, we cannot accept the similarity of the mutational pattern as a criterion of genetic identity.

MATHEMATICAL APPENDIX

I. Population dynamics.—

We wish to prove in this Appendix some of the quantitative statements made in the text. This will help to clarify their meaning and the limits of their applicability. The results are probably not new, the proofs perhaps somewhat simpler than those given previously. All equations refer to average values and are therefore of practical value only for the very high mutation rates where the fluctuations mentioned in the text are not the dominant feature.

Notation:

N = number of normal type bacteria at time t.

M = number of mutant type bacteria at time t.

a = forward mutation rate, defined as the fraction of normal type bacteria mutating per time unit.

b = reverse mutation rate, defined as the fraction of mutant type bacteria reverting to normal type per time unit.

k_N = growth rate constant of normal type bacteria, defined as the fraction of normal type bacteria which divide per time unit.

k = growth rate constant of mutant type bacteria, defined as above.

$s = k_N - k_M$ selection rate of normal against mutant type.

From these definitions follow the basic equations:

$$dN/dt = (k_N - a)N + bM$$

$$dM/dt = (k_M - b)M + aN$$

Case a.	*Case b.*
Forward mutation balanced by selection, no reverse mutation $b = 0$	Forward mutation balanced by reverse mutation, no selection $s = 0$, $k_N = k_M = k$

For these special cases the equations (1) simplify, respectively, to:

$$dN/dt = (k_N - a)N \qquad dN/dt = (k - a)N + bM$$
$$dM/dt = k_M M + aN \qquad dM/dt = (k - b)M + aN$$

By changing to logarithmic derivatives and simple transformations these equations are easily transformed to:

$$d(M/N)/dt = +a - (s - a)M/N \quad | \quad d[M/(M+N)]/dt = a - (a+b)M/(M+N)$$

The following integrals of these equations are adjusted to satisfy the initial condition $M = 0$ at $t = 0$:

$$\frac{M}{N} = \frac{a}{s-a}\left(1 - e^{-(s-a)t}\right) \quad | \quad \frac{M}{M+N} = \frac{a}{a+b}\left(1 - e^{-(a+b)t}\right)$$

In both cases the equilibrium values given in the text are found by putting t equal to infinity.

$$\left(\frac{M}{N}\right)_{equ.} = \frac{a}{s-a} \quad | \quad \left(\frac{M}{M+N}\right)_{equ.} = \frac{a}{a+b}$$

II. Probability of extinction of a type which mutates with a frequency "a" per generation, without reverse mutation.—

Let us assume that we start a culture with one individual of the normal type. The normal type may then become extinct by mutation of this individual before it divides; or, it may become extinct after the first division, if both offspring mutate before they in turn divide; or, if one of them mutates and the other divides and both its offspring later mutate, and so on.

We may ask what the total probability is that the clone generated by one bacterium will eventually contain none of the original type. It is obvious that the original type is certain to die out if the mutation rate is greater than .5 per generation, because then the net growth rate of the type is negative; that is, on the average the type loses more individuals by mutation than it gains by reproduction in each generation. The probability of extinction is therefore 1 for a mutation rate of .5 per generation, or any greater mutation rate.

However, also for mutation rates smaller than .5 per generation the type may die out by chance during the early stages of reproduction. The total chance may be calculated by a very simple argument. We wish to calculate the chance of extinction of the normal type in a clone which starts with one bacterium of the normal type, just after the birth of this bacterium. This chance will be a function of the mutation rate "*a*." Call the function $L(a)$. We calculate it by making use of the fact that any normal type offspring gives rise to its own sub-clone, and

that the chance of extinction within this sub-clone must be equal to the chance of extinction in the whole clone. This fact allows us to set up a simple equation for $L(a)$. Let us consider events up to the moment of the first division. Up to this time the chance of extinction is a, the chance of survival $1 - a$. In case of survival up to this point two normal type bacteria will be formed by division, and the chance of extinction for each of their sub-clones is again $L(a)$. The chance that both sub-clones will die out is, therefore, $L^2(a)$. The total chance that the whole clone dies out is, therefore,

$$L(a) = a + (1 - a)L^2(a)$$

This quadratic has two solutions, namely,

$L(a) = 1$ which is valid for a greater than .5

and $L(a) = a/(1 - a)$ which is valid for a smaller than .5

The complementary chance, that of survival of the type, is

$$S(a) = 1 - L(a) = (1 - 2a)/(1 - a)$$

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